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Purification, crystallization and preliminary X-ray analysis of the BRCT domains of human 53BP1 bound to the p53 tumour suppressor

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A complex of the DNA-binding domain of the tumour suppressor p53 bound to the BRCT domains of the p53-binding protein (53BP1) has been prepared and purified. Single crystals have been obtained using the microbatch technique with polyethylene glycol 4 kDa and ammonium sulfate. Crystals diffract X-rays to beyond 2.3 Å and belong to the space group $P2_12_12_1$. Several complete data sets have been collected from a number of crystals, each with different unit-cell parameters. Partial structures have been produced by successful placement of two copies of the p53 core region into the asymmetric unit. There is clear evidence for the binding protein and a complete structure determination is under way.

1. Introduction

DNA-repair proteins are highly diverse structurally but many have been shown to contain a conserved globular domain, first identified in the breast cancer tumour-suppressor protein BRCA1 and thus designated BRCT (BRCA1 C-terminus; Bork *et al.*, 1997; Callebaut & Mornon, 1997). The BRCT domain consists of approximately 95 amino-acid residues and has been found in 50 non-orthologous proteins (Bork *et al.*, 1997; Callebaut & Mornon, 1997) including major DNA-repair/cell-cycle related proteins. The crystal structures of the single BRCT domains of XRCC1 (Zhang *et al.*, 1998) and ligase III (Krishnan *et al.*, 2001) as well as the tandem repeat of BRCA1 (Williams *et al.*, 2001) have been reported. The BRCT domain consists of a four-stranded parallel β-sheet flanked by three α-helices.

The C-terminal region of the nuclear protein 53BP1 interacts with the DNA-binding core domain of the tumour-suppressor protein p53 and sequence analysis has shown that this region contains a BRCT tandem repeat (Iwabuchi *et al.*, 1998). Mutations in the p53 protein have been associated with human cancers and the vast majority of these mutations affect DNA binding (Cho *et al.*, 1994). It has been reported recently that after DNA damage, 53BP1 relocalizes to discrete nuclear foci that are thought to represent sites of DNA lesions and becomes hyperphosphorylated (Anderson *et al.*, 2001; Rappold *et al.*, 2001; Schultz *et al.*, 2000; Xia *et al.*, 2001). To investigate the nature of the 53BP1–p53 interaction, we have defined the minimal region of 53BP1, containing the tandem repeat of BRCT domains, which interacts directly with p53. Here, we report the crystallization of the DNA-binding domain of p53 in complex with the BRCT repeat of 53BP1. These crystals diffract X-rays to beyond 2.3 Å and several complete data sets have been collected to 2.6 Å. The core-domain structure of p53 (Cho *et al.*, 1994) has been successfully positioned into the complex structure and there is clear evidence for the position of 53BP1. A complete structure determination is under way. This structure will provide important insights into the molecular basis for protein–protein contacts mediated by BRCT domains.

2. Materials and methods

2.1. Cloning, expression and purification of the DNA-binding domain of p53

The p53 construct corresponding to the core domain (residues 94–312) was inserted into pRSET B (Invitrogen) using the *Nde*I and *Eco*RI enzyme sites. This region was subcloned from the yeast two-hybrid plasmid encoding human p53 residues 73–393 by PCR amplification using the primers 5′-GGGCCATATG TCA TCT TCT GTC CCT TCC CAG AAA ACC-3′ and 5′-GGGAATTCA GGT GTT GTT GGG CAG TGC TCG CTT AGT-3′. *Escherichia coli* cells, strain B834(DE3)plysS (Novagen), were transformed with this expression vector and grown at 310 K in Luria broth containing 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol. The cultures were induced at an OD_{600} (optical density at 600 nm) of 0.8, the culture was chilled, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.3 m*M* and $ZnSO_4$ added to a final concentration of 100 µ*M* and the induced culture allowed to grow overnight at 298 K. Cells were harvested by centrifugation (15 min at 6000*g*) and washed with 20 m*M* Tris–HCl pH 7.5. The

bacterial cell pellet was resuspended and sonicated in ion-exchange buffer *A* [50 m*M* Tris–HCl pH 7.5, 5 m*M* dithiothreitol (DTT), 2 m*M* ethylenediaminetetraacetic acid (EDTA), 200 µ*M* phenylmethylsulfonyl fluoride (PMSF) and 125 µ*M* benzamidine–HCl]. The cell lysate was subjected to centrifugation (at 39 000*g*) for 20 min to remove the cell debris and the supernatant was applied to a SP Sepharose Fast Flow (Amersham Biosciences) cation-exchange column pre-equilibrated with buffer *A*. Ion-exchange buffer *B* consists of 2 *M* NaCl in buffer *A*. Impurities were removed by washing with 150 ml of 8% buffer *B* (160 m*M* NaCl) and the column was washed with a linear gradient of 8–17% buffer *B* (160–340 m*M* NaCl) over 150 ml. Fractions containing p53 were pooled and diluted in a 1:2 ratio with buffer *A*. Further purification was achieved by loading p53 over Q-Sepharose onto a heparin-affinity column arranged in tandem. Both columns (pre-packed 5 ml Hi-Trap columns; Pharmacia) were pre-equilibrated with buffer *A*. Once loaded, the heparin column was isolated and washed with 15 ml of 8% buffer *B* (160 m*M* NaCl). Purified p53 was eluted from the heparin column using a linear gradient of 8–17% buffer *B* over 60 ml.

2.2. Cloning, expression and purification of the tandem BRCT domains of 53BP1

The p53-binding protein (hereafter referred to as BP1) was subcloned into the expression vector pRSET A utilizing the *Nhe*I and *Bam*HI sites. A number of constructs were subcloned from the plasmid pCMH6K53BP1 (Iwabuchi *et al.*, 1998). The optimal region of 53BP1, on the basis of protein solubility and the protein's ability to form a complex with p53, encoded residues 1722–1972 and included an amino-terminal histidine tag. For this construct, the primers 5′-GGGGCTAGCGGA CTC AAC AAG ACC TTG TTT CTG GGC TAC-3′ and 5′-GCGCGGATCC TTA GTG AGA AAC ATA ATC GTG TTT ATA-3′ were used. This clone contains the predicted BRCT tandem repeat reported to interact with p53 (Iwabuchi *et al.*, 1998). The BP1 expression vector was transformed into *E. coli* [strain B834(DE3)plysS] and cultured using the same procedure as described for p53-expressing cells. Induction was again performed once the culture reached an OD_{600} of 0.8, adding IPTG to a final concentration of 0.3 m*M*. The induced culture was allowed to grow overnight at 303 K. Cells were harvested, washed, sonicated and the lysate cleaned as for p53-expressing cells, except that nickel buffer *A* was used for lysis instead of ion-exchange buffer *A*. Nickel buffer *A* consists of 50 m*M* Tris–HCl pH 7.5, 30 m*M* imidazole, 300 m*M* NaCl, 5 m*M* β-mercaptoethanol, 200 µ*M* PMSF and 125 µ*M* benzamidine–HCl. Nickel buffer *B* replaces 30 m*M* imidazole with 300 m*M* imidazole. Lysate supernatant, containing BP1, was applied to an Ni–NTA agarose column (Qiagen) pre-equilibrated with nickel buffer *A*. The column was washed with sufficient nickel buffer *A* to allow the absorbance at 280 nm to approach the baseline. Protein was eluted with a single step to 100% buffer *B*. BP1-containing fractions were pooled, diluted in a 1:6 ratio with ion-exchange buffer *A* and loaded onto a 5 ml Hi-Trap Q-Sepharose column (Amersham Biosciences) for further purification. The column was washed with 15 ml of 5% buffer *B* (100 m*M* NaCl) and BP1 was eluted with a linear gradient of 5–20% buffer *B* in 60 ml.

2.3. Preparation and purification of the p53–BP1 complex

The p53–BP1 complex was prepared by direct mixing of the two purified protein components, with p53 being in a slight molar excess with respect to BP1. The mixture of p53 and BP1 (in a molar ratio of 1.1:1) was then dialyzed against gel-filtration buffer (50 m*M* Tris–HCl pH 7.5, 125 m*M* NaCl, 5 m*M* DTT, 2 m*M* EDTA, 200 µ*M* PMSF and 125 µ*M* benzamidine–HCl). The dialyzed complex was concentrated, typically to 3 mg ml^{-1}, using ultrafiltration concentrators (Amicon) with a 10 kDa molecular-weight cutoff. The complex was then purified by size-exclusion chromatography using a preparative Superdex-75 column (Amersham Biosciences).

2.4. Analytical methods

Protein purity was monitored by SDS–PAGE using Coomassie blue staining. Samples of both purified proteins, as well as a sample of dissolved crystals, were electroblotted onto Immobilin membrane (Amersham Biosciences) and the protein bands submitted for amino-terminal sequence analysis. Samples of both proteins were also analysed by electrospray mass spectrometry.

2.5. Crystallization

Crystal screening was performed using the microbatch method (Chayen *et al.*, 1990) with a sparse-matrix screening system (Jancarik & Kim, 1991). Crystals were grown under mineral oil (Sigma) using Terazaki microtitre plates (Greiner), mixing 1 µl of protein with an equal volume of a precipitant solution, and equilibrated at 287 K. Preliminary crystallization conditions were obtained using Crystal Screen I (Hampton Research). 2 µl drops containing a 1:1 ratio of protein to well solution were placed into the depression of the Terazaki plates.

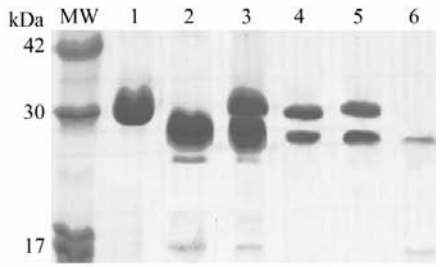
2.6. X-ray data collection

Crystals were subject to data collection at 110 K after first being flash-frozen in cryoprotection buffer [50 m*M* Tris–HCl pH 7.4, 100 m*M* $(NH_4)_2SO_4$, 30% PEG 4K and 15% glycerol]. X-ray diffraction data were collected using both a MAR345 image-plate scanner with a Cu $K\alpha$ rotating-anode source and the ADSC Quantum 4R CCD detector at the synchrotron light source at SRS, stations 9.6 and 14.2 (Daresbury, England). Data were processed using *MOSFLM*, *SCALA* and other programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

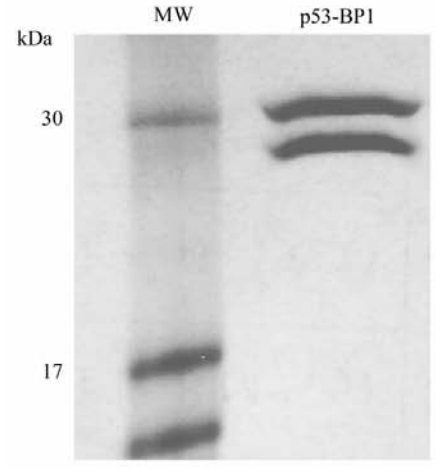
We have defined the minimal region of 53BP1, containing the tandem repeat of BRCT domains, which interacts directly with p53. We have cloned, overexpressed and purified the DNA-binding core region of p53 (residues 94–312). We expressed p53 at high levels (5–6 mg l^{-1}) in *E. coli* and readily purified the protein to homogeneity using routine chromatographic procedures (Fig. 1*a*). It has been reported that 53BP1 interacts with the DNA-binding domain of p53 *via* two C-terminal BRCT domains (Iwabuchi *et al.*, 1998). We produced a series of N-terminal truncated forms of 53BP1 in order to define the minimal interacting domain of 53BP1. These truncated proteins were expressed in *E. coli* and purified to homogeneity. We incubated the proteins with the recombinantly expressed DNA-binding domain of p53 and analysed the complexes by gel filtration (data not shown). A truncation consisting of the tandemly repeated BRCT domain region (residues 1722–1972) formed a very stable complex. Purified p53 (residues 94–312) had a mobility, as judged by SDS–PAGE, corresponding approximately to its predicted molecular weight of 24 500 Da (Fig. 1*a*). Amino-terminal sequence analysis confirmed the starting sequence as S(94)SSVPSQKT and mass spectrometry shows a single species of 24 121 Da. BP1 also gives a single product (29 987.7 Da) from mass spectrometry, although slightly heavier

than its predicted molecular weight of 29 950 Da (Fig. 1*a*). The amino-terminus was confirmed to be FRGSHHHHHHG, the polyhistidine-fusion region from the pRSETA vector. Size-exclusion chromatography in conjunction with amino-terminal sequence analysis suggests that both p53 and the BRCT domains of BP1 homodimerize: the BP1 dimer has a predicted mass of 59 900, the p53–BP1 complex a mass of 54 500 and the p53 dimer 49 100. Owing to the apparent lower molecular weight of p53 and the overlapping mobilities of the BP1 dimer and the complex, a slight molar excess of p53 was used during complex formation (Fig. 1*a*). Excess p53 dimer was easily removed by gel filtration (Fig. 1*a*).



(*a*)

(*b*)



(*c*)

Figure 1
(*a*) SDS–PAGE analysis of purified p53 (lane 1), 53BP1 (lane 2), dialysed p53–BP1 complex (lane 3), gel-filtered p53–BP1 complex (lanes 4 and 5) and excess p53 homodimer eluted from the gel-filtration column (lane 6). (*b*) Typical orthorhombic crystals of p53 in complex with the BRCT tandem repeat of 53BP1. (*c*) SDS–PAGE analysis of dissolved crystals confirms the presence of both p53 and BP1 proteins.

Table 1
Data collection.

Values in parentheses refer to the highest resolution shell. The space group is $P2_12_12_1$ and $\alpha = \beta = \gamma = 90.0^\circ$ for all crystals.

Crystal	D_{min} (Å)	R_{sym}	$I/\sigma(I)$	Completeness (%)	Multiplicity	a (Å)	b (Å)	c (Å)	V_M (Å^3 Da^{-1})	Solvent (%)
In-house	2.6	10.4 (35.8)	6.2 (1.4)	99.1 (96.9)	7.7 (3.7)	71.52	94.57	136.20	2.13	41.76
Dares-1	2.75	8.6 (26.8)	5.4 (2.7)	92.8 (91.0)	3.1 (3.1)	72.95	95.87	137.99	2.23	44.41
Dares-2	2.6	10.1 (62.2)	6.5 (1.1)	99.4 (95.9)	5.4 (4.5)	68.56	94.09	132.88	1.98	37.41
Dares-3	2.4	8.2 (39.3)	6.7 (1.9)	86.4 (52.3)	5.9 (4.5)	84.87	96.23	130.71	2.47	49.74

Crystallization experiments utilized the microbatch crystallization method (Chayen *et al.*, 1990) exploiting a sparse-matrix screening system (Jancarik & Kim, 1991) using Hampton Research Crystal Screens. The protein complex was dissolved in 10 m*M* Tris–HCl buffer pH 7.5 and 1 m*M* DTT at a concentration of 15 mg ml^{-1} prior to the crystallization experiments. Initial crystals grew from drops composed of 1 µl of protein (15 mg ml^{-1}) and 1 µl 200 m*M* $(NH_4)_2SO_4$ and 30% polyethylene glycol 4 kDa (PEG 4K) (condition No. 31 from Crystal Screen I solutions, Hampton Research) incubated overnight at 287 K under mineral oil. We optimized this crystallization condition by decreasing the protein concentration to 13 mg ml^{-1} and adding Tris–HCl buffer pH 7.5 to change the pH of the solution. Sharp rod-like crystals were obtained using 225–275 m*M* ammonium sulfate, 50 m*M* Tris–HCl pH 7.4, $(NH_4)_2SO_4$ and 20–25% PEG 4K, growing to approximately 0.3 × 0.07 × 0.07 mm at 287 K in 7–10 d (Fig. 1*b*). These crystallization conditions differ from those recently described for a similar BP1–p53 complex (Joo *et al.*, 2002). To confirm that the crystals were of the complex, we harvested several clusters of crystals, washed them with the crystallization buffer, dissolved the crystals in SDS buffer and analysed the solution by SDS–PAGE and amino-terminal sequencing. This confirmed that the crystals contained both p53 and BP1 proteins (Fig. 1*c*).

The crystals were unstable on exposure to X-rays and were mounted on loops and flash-frozen in liquid nitrogen at 110 K after a short soak in a harvesting solution. For the cryogenic experiments, a suitable cryoprotectant was determined to be reservoir solution plus 15%(*v*/*v*) glycerol. Successful flash-freezing was achieved when the crystals were transferred directly from the drop to cryoprotection solution and were allowed to equilibrate for 30 s. Data were collected at 110 K on a MAR345 image-plate detector using Cu $K\alpha$ radiation from an in-house Rigaku rotating-anode X-ray generator. Crystals of the p53–BP1 complex diffracted X-rays to beyond 2.6 Å and a 99.1% complete data set was obtained ('in-house' data in Table 1). As with all successive data sets from crystals of the complex, these belong to the orthorhombic space group $P2_12_12_1$. Crystals exhibited large variations in unit-cell parameters, with the in-house data set having a = 71.52, b = 94.57, c = 136.20 Å, $\alpha = \beta = \gamma = 90.0^\circ$. Three other 'native' data sets were collected, each from crystals taken to the synchrotron-radiation source at the Synchrotron Radiation Source (SRS, Daresbury, England) ('Dares-1', 'Dares-2' and 'Dares-3' in Table 1), with the best crystal diffracting to beyond 2.3 Å. An 86.4% complete data set to 2.4 Å was obtained from this last crystal, with unit-cell parameters a = 84.87, b = 96.23, c = 130.71 Å. In total four 'native' data sets (Table 1) and eight 'possible derivative' data sets (data not shown) have been collected. Assuming a 1:1 complex of p53 with BP1, supported by the dissolved crystal data (Fig. 1*c*), two copies of the complex per asymmetric unit gives realistic Matthews coefficient values (Matthews, 1968) for all data sets acquired (Table 1). Two p53 molecules per asymmetric unit cell (Fig. 2) were found in each data set by molecular replacement using *AMoRe* (Navaza, 1994) and coordinates of the p53 DNA-binding domain (PDB code 1tsr; Cho *et al.*, 1994). Rigid-body refinement (*REFMAC*) was conducted, giving final correlation coefficients of 47.59 and 44.57% for the 'in-house' and 'Dares-3' structures respectively. Broken density was observed for BP1 at this stage (Fig. 2). Although molecular-replacement trials with known BRCT domain structures (XRCC1, BRCA1 and ligase III; PDB codes 1cdz, 1jnx and

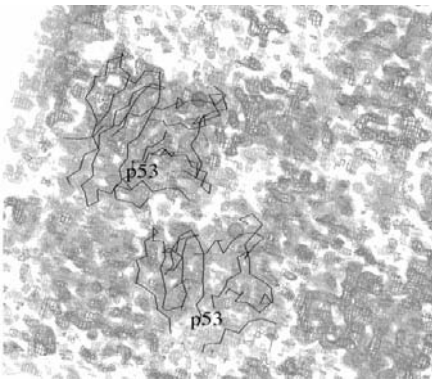


Figure 2
Two p53 molecules were found in each asymmetric unit by molecular replacement. Clear additional density, presumably for the BP1 molecules, can be observed in preliminary electron-density maps.

1imo, respectively) using a variety of programs, including *AMoRe* (Navaza, 1994), failed to solve the structure of the BRCT domains of BP1, they did position the BRCT 53BP1 trial structures consistently into this region of broken density.

Solvent flattening and noncrystallographic symmetry averaging were conducted, with extensive phase improvement from 6.0 Å, using *DM* (Collaborative Computational Project, Number 4, 1994). Initially arbitrarily orientated BRCT domains placed into the broken density were used to define molecular masks for the two BRCT domains of BP1. Multicrystal averaging was then performed using *DMULTI* (Collaborative Computational Project, Number 4, 1994) over the four 'native' structures. This enabled the approximate orientation of a C^{α} trace of the BRCT domains onto 'skeletonized' density (generated by *XSKEL* in *XTALVIEW*; McRee, 1999). A repeat cycle of density modification of each crystal structure with multiple domains together with cross-crystal averaging has resulted in sufficient improvement in the electron density for interpretation to commence (Fig. 2). A description of the structure and its biological implications will be discussed elsewhere when the structure-determination process has been completed.

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